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Glucocorticoids Protect Against the Delayed Behavioral and Cellular Effects of Acute Stress on the Amygdala

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Abstract

Background—A single episode of acute immobilization stress has previously been shown to trigger a delayed onset of anxiety-like behavior and spinogenesis in the basolateral amygdala (BLA) of rats. Spurred on by a seemingly paradoxical observation in which even a modest increase in corticosterone (CORT), caused by a single vehicle injection before stress, could dampen the delayed effects of stress, we hypothesized a protective role for glucocorticoids against stress.

Methods—We tested this hypothesis by analyzing how manipulations in CORT levels modulate delayed increase in anxiety-like behavior of rats on the elevated plus-maze 10 days after acute stress. We also investigated the cellular correlates of different levels of anxiety under different CORT conditions by quantifying spine density on Golgi-stained BLA principal neurons.

Results—CORT in drinking water for 12 hours preceding acute stress prevented delayed increase in anxiety rather than exacerbating it. Conversely, vehicle injection failed to prevent the anxiogenic effect of stress in bilaterally adrenalectomized rats. However, when CORT was restored in adrenalectomized rats by injection, the delayed anxiogenic effect of stress was once again blocked. Finally, high and low anxiety states were accompanied by high and low levels of BLA spine density.

Conclusions—Our findings suggest that the presence of elevated levels of CORT at the time of acute stress confers protection against the delayed enhancing effect of stress on BLA synaptic connectivity and anxiety-like behavior. These observations are consistent with clinical reports on the protective effects of glucocorticoids against the development of posttraumatic symptoms triggered by traumatic stress.

Keywords

Animal models; basolateral amygdala; anxiety; dendritic spines; glucocorticoids; posttraumatic stress disorder

We previously reported that a single 2-hour episode of immobilization stress leads to a delayed increase in anxiety-like behavior that is paralleled by an increase in spine-density on basolateral amygdala (BLA) principal neurons (1). Interestingly, the increase in anxiety and BLA spine-density was evident only at 10 days after acute stress and not 1 day after. These

effects are reminiscent of posttraumatic stress disorder (PTSD) in which a single, traumatic event triggers changes in anxiety and other behaviors that are both delayed and prolonged (2,3) and accompanied by amygdalar hyperactivity (4). Thus, our rodent model is attractive in relation to PTSD because it leads to a gradual buildup of anxiety that is manifested well after the acute stressor and is accompanied by cellular changes in a brain structure implicated in the emotional symptoms of PTSD (5).

PTSD is a psychiatric condition that occurs in the aftermath of a traumatic incident and is characterized by persistent flashbacks, hypervigilance, and hyperanxiety (2). Whereas traditional laboratory approaches to study this disorder used rodent models of chronic stress to assess the immediate effects of stress, more recent studies have also examined the delayed effects of acute stress (1,6). Furthermore, although the hippocampus was the primary focus of earlier studies, recent research, such as that using animal models of predator stress, highlights the importance of the amygdala (7,8). This is of particular relevance because the amygdala plays a pivotal role in processing emotional memories (9), and dysregulation in amygdalar function could form the basis for flashbacks and high anxiety observed in patients (10–12).

A somewhat counterintuitive feature of PTSD comes from clinical reports that individuals having lower levels of cortisol are more susceptible to developing PTSD (13). Furthermore, patients who receive stress levels of cortisol as part of their treatment in intensive care units (ICU) have a lower probability of developing ICU-related PTSD symptoms (14,15). These observations have given rise to the idea that below-normal cortisol levels may render individuals susceptible to PTSD and that glucocorticoids may protect against its development (13,16–18). Preclinical studies have also suggested the involvement of glucocorticoids in the hypothalamic-pituitary-adrenal (HPA) axis response and pathogenesis of anxiety (19,20). We tested this idea by subjecting a rodent model of acute stress to two challenges. First, we tested whether supplementing corticosterone (CORT) in drinking water before stress prevents the delayed increase in anxiety-like behavior. Second, in a complementary strategy, we tested whether depletion by adrenalectomy and replacement of CORT exacerbates or alleviates the delayed anxiogenic effects of acute stress. Finally, we probed the cellular correlates of these behavioral effects by testing if the same manipulations also prevent the delayed increase in BLA spine-density.

Methods and Materials

Animals

Male Wistar rats (45–60 days old, 250–300 g) were housed in a 14/10-hour light/dark schedule (lights on at 8 AM) with ad libitum access to food and water at the National Centre for Biological Sciences, Bangalore, India. The Institutional Animal Ethics Committee approved all procedures related to animal maintenance and experimentation.

Stress

Rats were subjected to a single, 2-hour episode of acute immobilization stress (1) between 10 AM and 12 PM in plastic immobilization bags (with no access to food or water) in a room different from that used for housing. After stress, animals were returned to home cages and subjected to behavioral/morphologic analysis 10 days after acute stress.

Elevated Plus Maze (EPM)

Animals were placed in the center of the maze facing a closed arm and allowed to explore the maze for 5 min each. All trials were conducted between 10 AM and 2 PM and videotaped for offline analysis. Number of entries and time spent in open and closed arms

was analyzed while being blind to the treatment. Animals that did not explore either open or closed arms were excluded from analysis. Open-arm exploration was measured by normalizing to total exploration on the maze and anxiety scored for as a reduction in open-arm exploration. Anxiety Index, a measure taking into account both open-arm time and entries, was calculated as previously described (21).

Surgery

Animals were subjected to bilateral adrenalectomy under ketamine and xylazine anesthesia. Sham-operated animals underwent an identical surgical procedure without removing adrenal glands. During recovery (5–7 days) and subsequent experimental procedures, animals were given .9% NaCl in drinking water to maintain sodium balance.

Glucocorticoid Treatment

CORT (Sigma) was either injected subcutaneously in the neck scruff (40 mg/kg, in vehicle containing propylene glycol and DMSO, 9:1) (22) or administered through drinking water (400 µg/mL dissolved in 2.4% ethanol, for 12 hours overnight (7:30 PM–7:30 AM) (23). These dosages have been demonstrated to produce hippocampal dendritic remodeling, which is preventable by treatment with phenytoin and antidepressants (24,25), and the effects of these dosages are comparable to that of chronic restraint stress (26) and are reversible. Dexamethasone was injected subcutaneously (.25 mg/kg, in propylene glycol) 4 hours before subjecting rats to stress (28).

Radioimmunoassay

To avoid stressful effects of tail bleeding, animals used for behavioral experiments were not subjected to blood sampling. In a separate set of animals, trunk blood CORT levels were estimated using Coat-A-Count Rat Corticosterone kit (Diagnostic Products Corporation). Inter- and intraassay coefficients of variation were less than 10%.

Dendritic Spine-Density Analysis

Golgi-stained brains were coded and processed (Supplement 1). Coronal sections (120 µm) containing BLA (Bregma –1.92 to –3.12 mm) (27) were used to visualize medium spiny pyramidal neurons. An experimenter blind to treatment conditions quantified spine-density. All protrusions (irrespective of morphological characteristics) on primary dendrite were counted as spines along an 80-µm stretch using NeuroLucida (MicroBrightField) attached to an Olympus BX61 microscope (100×, 1.3 NA).

Statistical Analysis

Values are reported as mean ± SEM, *n* refers to number of neurons analyzed for morphometry, and *N* represents number of rats used. Data were analyzed with one-way analysis of variance (ANOVA), and post hoc Bonferroni test was used for multiple comparisons with significance levels set at *p* < .05. Statistical analyses were performed using Prism 5 (Graphpad Software).

Supplement 1 provides more details about methods and materials used in this study.

Results

Injection of Vehicle Alone Alleviates the Delayed Anxiogenic Effect of Acute Stress

As reported previously (1), a single 2 h episode of immobilization stress (Fig. 1A) leads to enhanced anxiety-like behavior 10 days later on the EPM. This delayed anxiogenic effect was exhibited as a reduction in both time spent (Figure 1B) and number of open-arm entries

(Figure S1A in Supplement 1). Surprisingly, a single subcutaneous injection of vehicle 30 min before the same acute stress (Figure 1A, Veh inj + Stress) reversed the delayed increase in anxiety back to control levels for time (Figure 1B) and entries in open-arm (Figure S1A in Supplement 1). However, the same vehicle injection, given in the absence of stress (Figure 1A, Veh inj), had no significant effect on open-arm exploration 10 days later (Figure 1B, and S1A in Supplement 1). Closed-arm entries were not affected by any of these treatments, ruling out deficits in locomotor activity (Figure S1B in Supplement 1). Thus, vehicle injection that on its own did not affect anxiety was nonetheless effective in preventing the anxiogenic effects of acute stress 10 days later.

To investigate this unexpected protective effect of vehicle injection, we compared its impact to that of 2 hours of stress. Radioimmunoassays show an approximately 10-fold increase in circulating levels of CORT at the end of stress (Figure 1C). In contrast, vehicle injection alone caused a 2-fold increase after 30 min (Figure 1D) and returned to basal levels after 150 min (Figure 1D), which is consistent with the expected shutoff of the HPA axis. Interestingly, in animals subjected to 2-hour stress at the same 30-min time point after vehicle injection, the two manipulations added up to CORT levels comparable to those at the end of 2-hour stress alone (Figure 1E). Thus, the difference in anxiety between animals subjected to stress only versus stress preceded by vehicle injection cannot be explained by CORT levels at the end of stress. The difference instead appears to lie in CORT levels just before the start of acute stress. This raises the intriguing possibility that injection-induced increase in CORT preceding acute stress protects against the later development of anxiety.

Prior Exposure to Glucocorticoids Prevents the Delayed Anxiogenic Effects of Acute Stress

Next we examined if a more robust elevation in prestress gluco-corticoid levels through direct experimental manipulation, rather than the serendipitous effect of vehicle injection, prevents the subsequent increase in anxiety-like behavior. Hence, we administered rats with CORT through drinking water for 12 hours overnight before the 2-hour stress next morning (Figure 2A). In a separate set of animals, we determined that this dose of CORT in drinking water elevates circulating CORT to near stress levels (373.3 ± 12.0 ng/mL, $N=3$ vs. 474.2 ± 51.2 ng/mL, $N=6$ immediately after acute stress), which was significantly higher than levels in vehicle-treated animals (66.5 ± 16.8 ng/mL, $N=3$) receiving 2.4% ethanol for 12 hours in drinking water. In these CORT-fed animals, acute stress failed to increase anxiety after 10 days (Figure 2B). There was no significant difference in open-arm time (Figure 2B) or entries (Figure S2A in Supplement 1) between unstressed control and CORT-treated animals 10 days after stress. In contrast, animals fed with vehicle before stress exhibited levels of open-arm exploration not significantly different from reduced levels seen 10 days after stress alone (Figure 2B, and S2A in Supplement 1). Again, there was no difference in number of closed-arm entries (Figure S2B in Supplement 1). In summary, animals subjected to 12 hours of glucocorticoid exposure followed by 2 hours of acute stress quite remarkably showed anxiety levels that were comparable to unstressed animals.

Depletion of Glucocorticoids Exacerbates the Delayed Anxiogenic Effects of Acute Stress

Having observed a protective role for glucocorticoid exposure before stress in the development of anxiety-like behavior, we asked whether the converse were also true: would absence or shortfall of glucocorticoids have the opposite effect? We addressed this question by subjecting adrenalectomized (ADX) rats to the same manipulations of vehicle injection and acute stress and analyzed anxiety-like behavior 10 days later. Surgical procedures used for adrenalectomy (Figure 3A) had no impact on the efficacy of acute stress in reducing time spent (Figure 3B) and entries (Figure 3C, black and red dotted lines) in open-arm in sham-operated animals. Indeed, reduced level of open-arm exploration achieved in sham-operated

animals was not significantly different from those seen in intact animals after stress (Figure 3B, red dotted line).

Next, we tested whether vehicle injection is still able to confer its protective effect against stress-induced anxiety in the absence of glucocorticoids. We subjected ADX animals to the same manipulation (ie, vehicle injection) 30 min before acute stress (ADX-Veh inj + Stress, Figure 3A). Despite prior vehicle injection, acute stress triggered delayed increase in anxiety in ADX animals, manifested by a significant reduction in open-arm exploration (Figure 3C, D). These reduced values were in stark contrast to intact animals subjected to vehicle injection before stress (Veh inj + Stress from Figure 1B, replotted in Figure 3C, D for comparison) suggesting that the protective effect of vehicle injections was indeed due to CORT because the injection-associated CORT increase is absent in the ADX animals.

Restoration of Glucocorticoids in Adrenalectomized Animals Prevents the Delayed Anxiogenic Effects of Acute Stress

As a final test of the efficacy of glucocorticoids, we attempted to rescue its protective effect by injecting CORT into ADX rats and examined if this replacement would block the delayed anxiogenic effect of acute stress. Indeed, ADX animals injected with CORT 30 min prior to stress (ADX - CORT inj + Stress) displayed lower levels of anxiety-like behavior relative to ADX animals without CORT replacement. This beneficial effect of CORT replacement in stressed ADX rats was manifested as a significant increase in open-arm entries (Figure 3C) and a similar trend in open-arm time (Figure 3D); closed-arm entries were not significantly different (Figure S3A in Supplement 1).

This conclusion is further supported by our finding that treating adrenally intact rats with dexamethasone (DEX), which does not readily enter the brain (28–30), reduced CORT levels to below basal levels and increased anxiety-like behavior 10 days later (Figure S4 in Supplement 1).

Prior Exposure to Glucocorticoids Prevents Delayed Spinogenesis in BLA After Acute Stress

The acute stress paradigm used here also leads to a delayed increase in spine density in BLA principal neurons at the same 10-day time point when anxiety-like behavior is increased (1). The correlation between enhanced anxiety and BLA spinogenesis is further strengthened by accumulating evidence that behavioral or genetic manipulations that trigger BLA spinogenesis also enhance anxiety in rodents (31,32). This gives rise to the rather counterintuitive prediction that despite two successive manipulations that elevate CORT levels, BLA neurons in these animals should have spine densities as low as unstressed animals.

To test this prediction, spine density was quantified on primary dendrites of BLA pyramidal neurons of rats fed with CORT or vehicle in drinking water before immobilization (Figure 2A and Figure 4B; Figure 4A shows locations of individual neurons). In agreement with earlier reports, acute stress caused a delayed increase in BLA spine density (Figure 4C, red squares), and strikingly, this was prevented by prior administration of CORT (Figure 4C, green squares). On the other hand, vehicle treated animals (Figure 4C, black unfilled squares) showed BLA spine numbers similar to stressed animals. Detailed segmental analysis of spine density along the dendritic length showed that this prevention of delayed spinogenesis by prior CORT treatment was prominent in the proximal part of the dendrite (Figure 4C). Suppression of stress-induced spinogenesis was significant in proximal dendrites (Figure 4D, top), and was also apparent further along distal dendrites (Figure 4D, bottom). Significantly, treatment of animals with vehicle or CORT alone (for 12 hours) did

not lead to a delayed increase in spine numbers (Figure S5 in Supplement 1), suggesting that although CORT alone was not capable of resulting in spine changes, its administration before stress prevents spinogenesis.

Depletion of Glucocorticoids Leads to Stress-Induced Spinogenesis and This Is Reversed by Restoration of Glucocorticoids

Next, we wanted to test whether CORT pretreatment in ADX animals would be able to block stress-induced spinogenesis. To this end, spine density was quantified in BLA pyramidal neurons in animals from various treatment groups (Figure 3A; see Figure 5A for locations of individual neurons and Figure 5B for representative images). Segmental analysis revealed that stress also caused an increase in spine density in sham-operated animals (Figure 5C), with proximal dendrites exhibiting the most robust effect (Figure 5D, top). ADX animals that were subjected to vehicle injection before stress also showed a similar increase in spine numbers. Conversely, CORT injection before stress in ADX animals prevented BLA spinogenesis (Figure 5D, top) such that proximal spine density in these animals was not significantly different from sham-operated controls. In contrast, spine density on distal dendrites remained unchanged across the various treatment groups (Figure 5D, bottom). On the other hand, animals that were subjected to ADX alone did not show a delayed increase in spine numbers and were comparable to controls (Figure S6 in Supplement 1), suggesting that the protective effect on spinogenesis was due to CORT treatment before stress in ADX animals.

High and Low Levels of Anxiety Are Accompanied by High and Low Levels of BLA Spine Density

Based on the behavioral and morphologic findings described so far, we find that under a diverse range of conditions involving intact, sham-operated, and ADX rats, whenever acute stress caused an increase in BLA spine density, it was accompanied by enhanced anxiety-like behavior. Figure 6 summarizes these combined results on the effects of acute stress at both cellular and behavioral levels by depicting the positive relationship between a higher anxiety index (Anxiety, upward arrow) and elevated levels of BLA spine density (Spinogenesis, rightward arrow). Whereas vehicle-treated stressed animals exhibit anxiety indices and spine numbers that are as high as stressed animals (Figure 6A, red and white squares), CORT treatment before stress in intact animals (Figure 6A, green squares) brings spine density and anxiety indices closer to control levels (Figure 6A, black triangles). Similarly, despite being subjected to stress, ADX animals with CORT injections exhibit BLA spine density and anxiety indices (Figure 6B, ADX-CORT inj + Stress, green squares) as low as Sham-Control animals (Figure 6B, black triangles). In contrast, vehicle injection fails to protect ADX animals against stress-induced increase in anxiety and spinogenesis (Figure 6B, white squares), as a result of which these measures are comparable to those seen in sham-operated animals subjected to stress (Figure 6B, red squares). Thus, high and low anxiety indices were always accompanied by high and low levels of BLA spine density, respectively, suggesting a link between BLA spinogenesis and enhanced anxiety-like behavior.

Discussion

While earlier studies on the effects of stress on the brain were largely focused on the hippocampus, more recently developed animal models of PTSD have investigated other brain regions (6,33,34), and the literature has been extensively reviewed (5,35). This study was motivated by clinical observations on two facets of PTSD— one temporal and the other therapeutic. First, although PTSD can be triggered by a single severely stressful event, some components of the affective symptoms persist well beyond the original trauma. Here we

have tried to capture the essence of this temporal feature by building on a rodent model of acute stress, which triggered higher anxiety-like behavior and formation of dendritic spines in the BLA not 1 but 10 days later (1). Second, another striking feature of PTSD emerges from clinical reports that cortisol treatment reduces the development of the cardinal symptoms of PTSD (14,36–39). Here, we probed this somewhat counterintuitive observation by testing whether glucocorticoids indeed prevent the delayed impact of stress in rats. In our study, an indication of a potentially protective effect came from the serendipitous finding that a modest CORT increase elicited by vehicle injection before stress protects against subsequent development of anxiety. This is in agreement with an earlier report (21).

We built on this result through three manipulations of CORT levels. First, an excess of CORT in drinking water before acute stress blocked the delayed increase in anxiety. Second, we used hormone depletion and replacement experiments to further examine this phenomenon. Strikingly, in ADX rats, vehicle injection failed to block the delayed anxiogenic effects of stress. However, the protective effect was reinstated when ADX rats were injected with CORT. Third, stress following treatment with DEX, which markedly reduced CORT levels below baseline via actions at the pituitary but is known not to readily enter the brain (29,30), led to anxiety-like behavior 10 days later. If DEX were getting into the brain and, as expected, acting as a GR agonist, we would have expected it to block the stress effect, like CORT. Another alternative interpretation of this finding is that, even if some DEX enters the brain, DEX does not activate mineralocorticoid receptors (29,40), and thus MR might be involved in the protective effects of CORT shown here.

Finally, we explored the cellular correlate of these protective effects based on earlier observations that stress elicits BLA spinogenesis at the same 10-day time point when higher anxiety is observed (1). We find that every manipulation of CORT that prevented the anxiogenic effects was also effective in suppressing growth of BLA spines, thereby demonstrating a strong link between stress-induced spinogenesis in the amygdala and high anxiety.

These findings are reminiscent of the occurrence of PTSD in individuals with below normal levels of cortisol at the time of the traumatic event (13). Clinical studies have supported this by showing that supplemental cortisol given to people with below normal cortisol levels reduces the incidence of PTSD-related symptoms caused by septic shock and major cardiac surgery (14,36–38,41). Also, it was demonstrated that glucocorticoids could reduce phobic fear (39). Notably, human subjects given cortisol infusions immediately after traumatic stress show a significant reduction of PTSD symptoms (42). In the same study, glucocorticoid treatment in a rodent model of predator stress prevented dendritic debranching in the dentate gyrus (42).

In this study, it was a modest elevation of CORT at the time of acute stress that reversed the delayed increase in anxiety and BLA spine density. Here, a relatively large dose of CORT was able to prevent delayed anxiety and spine growth, indicating it is not dose but timing of CORT elevation that may be a key determinant. In contrast, a single large dose of CORT has been shown earlier to mimic the effects of repeated immobilization stress by increasing anxiety and dendritic branching of BLA neurons (43). Although the mechanism for this effect remains to be explored, it should be noted that spine density was not assessed in that study. Acute immobilization stress, on the other hand, does not cause dendritic hypertrophy 10 days later, but only spinogenesis (1). Thus, it is both dose and timing of glucocorticoid elevation that differentiates the contrasting outcomes, along with the dissociation between changes in spines and dendrites. Our findings provide a model to explore mechanisms for the protective, albeit counterintuitive, role for glucocorticoids against the delayed impact of acute stress on the amygdala at both cellular and behavioral levels. Interestingly, a similar

observation has been reported earlier in the hippocampal CA3 region in which a combination of repeated stress and orally administered CORT failed to elicit dendritic atrophy, whereas stress alone or daily CORT injections alone each caused atrophy (44).

Although little is known about cellular processes that mediate delayed amygdalar spinogenesis following acute stress, recent studies suggest possible mechanisms for the acute, protective effects of CORT. Brief but severe stress is known to cause a surge of high CORT and glutamate release in amygdala (45). This elevation of glutamate could trigger plasticity mechanisms at excitatory synapses eventually culminating in structural plasticity of spines (32). For instance, there is a role for NR2A receptors (46) and elevated brain-derived neurotrophic factor (47) during a time window after acute stress, when a modest elevation of CORT might counteract these influences and lead to the outcome seen here. A recent study showed rapid actions of glucocorticoids, acting via both MR and GR, on spine formation and downregulation, that provide the basis for future studies of the effects of timed CORT elevations on dendritic spine dynamics (48). Moreover, in vitro application of stress levels of CORT reduces gamma-aminobutyric acid–ergic inhibitory transmission but enhances intrinsic excitability of principal neurons in BLA slices (49). Stress-induced disruption in the excitatory/inhibitory balance (possibly by the rapid, nongenomic actions of CORT to stimulate endocannabinoid release) may cause a gradual strengthening of BLA synaptic connectivity (50).

It is interesting to note that spine density on dendrites more proximal to soma appears to be the best predictor of high and low anxiety levels (Figure 6). These proximal excitatory inputs are likely to have greater influence on firing output of BLA projection neurons, which in turn could modulate the functional output of the amygdala in terms of fear and anxiety (51). These and related possibilities await further investigation.

Perhaps the most striking aspect of our results is that two successive manipulations that both elevate CORT levels by themselves together reset BLA spine densities to unstressed, control levels. Moreover, these high and low levels of BLA spine density appear to be reliable predictors of high and low anxiety states, respectively (Figure 6). Thus, the delayed impact of acute stress in the amygdala may vary with the presence or absence of adequate levels of CORT prior to stress. This is reminiscent of a report on metaplasticity in the amygdala wherein one pulse of in vitro CORT caused a rapid, long-lasting increase in glutamatergic transmission in BLA neurons, but prior exposure to restraint stress or another CORT pulse suppressed this effect (52). Together, these findings from animal models provide a useful framework for examining the cellular and molecular changes in the amygdala that gradually develop after the initial endocrine and physiologic response triggered during, and immediately after, severe stress. Analysis of this progression of the initial stress response into a psychiatric condition is likely to provide valuable insights into possible therapeutic interventions after the initial traumatic event.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Injection (inj) of vehicle (Veh) (and its associated corticosterone [CORT] increase) alleviates the delayed anxiogenic effect of acute stress. **(A)** Schematic representation of experimental protocol in which male Wistar rats were subjected to a single episode of acute immobilization stress (2 hours) and tested for anxiety-like behavior after 10 days on elevated plus maze in parallel with unstressed controls. In addition, rats that were subjected to Veh inj 30 min before acute stress or were injected with vehicle alone were also tested for anxiety-like behavior. **(B)** Stress leads to a reduction in the time spent (% open-arm time, Control: 58 ± 2.6 , $N=10$; Stress: 39 ± 3.1 , $N=10$). A single subcutaneous inj of vehicle 30 min before the same stress reversed the delayed increase in anxiety-like behavior to control levels (Veh inj + Stress: 55 ± 5.6 , $N=9$). However, the same Veh inj given in the absence of stress had no effect on open-arm exploration 10 days later (Veh inj: 58.8 ± 2.5 , $N=10$). Significant difference between groups are indicated as * $p < .05$ and ** $p < .01$. **(C)** Radioimmunoassays (performed on blood samples collected at various time points indicated by red arrows) showed that there was an approximately 10-fold increase in circulating levels of CORT at the end of the 2-hour stress episode (Stress: 474.2 ± 51.2 ng/ml, $N=6$; Control: 48.2 ± 8.3 ng/mL, $N=6$). **(D)** Veh inj alone caused a relatively modest twofold increase after 30 min (106.2 ± 25.1 ng/mL, $N=6$) and when measured 150 min after the Veh inj, CORT levels were comparable to basal values (43.3 ± 3.8 ng/mL, $N=6$). **(E)** CORT levels in animals that were subjected to stress 30 min after the Veh inj (Veh inj + Stress: 541.7 ± 44.9 ng/mL, $N=6$) was not significantly different from the levels reported in panel C at the end of the stress episode. ****Significant difference between groups, $p < .0001$.

Figure 2.

Prior exposure to glucocorticoids prevents the delayed anxiogenic effects of acute stress. **(A)** Schematic representation of treatment regime where in rats were fed with either corticosterone (CORT; 400 $\mu\text{g/mL}$) in/or vehicle (Veh; 2.4% ethanol) for 12 hours before being subjected to a single episode of acute stress. Delayed onset of anxiety-like behavior was tested after 10 days on elevated plus maze in parallel with control and stressed rats. **(B)** Prior administration of CORT prevented the delayed increased in anxiety-like behavior and time spent in open-arm was comparable to those of control animals which were not subjected to stress (% open-arm time, CORT oral + Stress: 60.7 ± 3.2 , $N = 13$; Control: 65.2 ± 4.7 , $N = 10$, p value, ns). Although CORT-fed stressed animals exhibited a greater degree of open-arm exploration compared with vehicle-fed stressed animals (Veh oral + Stress: 52 ± 4.8 , $N = 10$), they were significantly different from animals that were only subjected to stress (Stress: 45.1 ± 5.6 , $N = 13$). Significant difference between groups is indicated by $*p < .05$ and $**p < .01$.

Figure 3.

Depletion of glucocorticoids exacerbates the delayed anxiogenic effects of acute stress, and this is alleviated by restoration of glucocorticoids. **(A)** Schematic representation of experimental protocol. To control for the effects of surgery, rats were sham-operated and allowed to recover for 5 to 7 days before being subjected to acute stress. Delayed onset of anxiety-like behavior was measured 10 days later along with sham controls that were not subjected to stress. Bilaterally adrenalectomized (ADX) animals were similarly allowed to recover postsurgery and subjected to either a vehicle (Veh) or CORT injection (inj) 30 min before acute stress. There were also tested for anxiety-like behavior 10 days later along with the previously mentioned sham operated animals. **(B)** Stress leads to a significant reduction in open-arm exploration in sham-operated animals when compared with corresponding controls (% open-arm time, Sham-Control: 57 ± 5.6 %, $N=9$; Sham-Stress: 36.7 ± 4.2 %, $N=11$). This was similar to the levels of anxiety observed in intact animals that were subjected to stress as indicated by the dotted black and red lines (Control and Stress, plotted from mean values obtained in Figure 1B). **(C)** Administration of CORT to ADX animals before stress leads to a significant reduction in anxiety-like behavior as observed by an increase in the number of entries in open arm (% open-arm entries, ADX-Veh inj + Stress: 45.3 ± 6.7 %, $N=8$; ADX-CORT inj + Stress: 63.1 ± 7.4 %, $N=8$) to levels comparable to Veh inj before stress in intact animals (Veh inj + Stress: 59.3 ± 4.6 %, $N=9$, replotted from Figure S1A in Supplement 1). *Significant difference between groups, $p < .05$. **(D)** Veh inj does not provide protection from delayed increase in anxiety-like behavior in ADX animals as observed by time spent in open arm (% open-arm time, ADX-Veh inj + Stress: 33.6 ± 6.8 %, $N=8$), which was significantly different when compared with the same Veh inj before stress in intact animals (Veh inj + Stress: 55 ± 5.6 %, $N=9$, replotted from Figure 1B). Furthermore, when the ADX animals were injected with CORT 30 min before stress, there was a trend toward the reduction of anxiety (ADX-CORT inj + Stress: 48.8 ± 7.3 %, $N=8$). To compare these values to the relevant controls, means values from Figure 3B are replotted as dotted black and red lines (Sham-Control and Sham-Stress respectively).

Figure 4.

Prior exposure to glucocorticoids prevents delayed spinogenesis in the basolateral amygdala (BLA) after acute stress. **(A)** Locations of medium spiny pyramidal neurons that were analyzed for spinogenesis in BLA (reprinted from Paxinos and Watson [27] with permission from Elsevier, copyright 2007). **(B)** Representative images of primary dendrites of BLA pyramidal neurons (40 $\times$, scale bar: 10 μ m). **(C)** Segmental analysis of mean numbers of spines in each successive 10- μ m segment of the primary dendrite as a function of the distance of that segment from the origin of the branch. Significant differences observed in specific segments are indicated by either * (for Control vs. Stress) or ¥ (for vehicle [Veh] oral + Stress vs. CORT oral + Stress) as follows: * p < .05, ** p < .01, *** p < .001, **** p < .0001, ¥ p < .05, ¥¥ p < .001. **(D)** Stress leads to an increase in spinogenesis in the proximal 40 μ m of the dendrite (top panel, total number of spines, Control: 59.6 $\pm$ 1.8, n = 24, N = 4;

Stress: 72.1 ± 1.4 , $n = 25$, $N = 4$). Veh-treated animals that were subjected to stress show levels comparable to stress alone while in CORT fed animals were comparable to controls despite being stressed (Veh oral +Stress: 73.1 ± 1.7 , $n = 24$, $N = 4$; CORT oral +Stress: 62.2 ± 1.9 , $n = 24$, $N = 4$). A similar effect was also observed in the distal 40 μm of the dendrite (bottom panel, Control: 59.9 ± 1.3 , $n = 24$, $N = 4$; Stress: 68.5 ± 1.5 , $n = 25$, $N = 4$; Veh oral +Stress: 71.4 ± 1.6 , $n = 24$, $N = 4$; CORT oral +Stress: 64.8 ± 1.5 , $n = 24$, $N = 4$). Significant differences are indicated as follows: $*p < .05$, $***p < .001$, $****p < .0001$.

Figure 5.

Depletion of glucocorticoids leads to stress-induced spinogenesis, and this is reversed by restoration of glucocorticoids. **(A)** Locations of medium spiny pyramidal neurons that were analyzed for spinogenesis in basolateral amygdala (BLA) (reprinted from Paxinos and Watson [27] with permission from Elsevier, copyright 2007). **(B)** Representative images of primary dendrites of BLA pyramidal neurons (40×, scale bar: 10 μm). **(C)** Segmental analysis of mean numbers of spines in each successive 10-μm segment of the primary dendrite as a function of the distance of that segment from the origin of the branch. Significant differences observed in specific segments are indicated by either * (for Sham Control vs. Sham Stress) or ¥ (for adrenalectomized [ADX]–vehicle-injected [Veh inj] + Stress vs. ADX–CORT inj + Stress) as follows: * $p < .05$, ** $p < .01$, ¥ $p < .01$. **(D)** Stress causes an increase in spinogenesis in the proximal 30 μm of the dendrite (top panel, total no. of spines, Sham-Control: 46.3 ± 1.2 , $n = 25$, $N = 4$; Sham-Stress: 53.3 ± 1.3 , $n = 24$, $N = 4$). Glucocorticoid-depleted animals also displayed a similar delayed increase in spine numbers when subjected to stress after a Veh inj (ADX–Veh inj + Stress: 51.5 ± 1.4 , $n = 24$, $N = 4$), whereas prior administration of CORT to ADX animals prevented the delayed increase in spinogenesis and restored the spine numbers comparable to Sham–Control animals (ADX–

CORT inj +Stress: 46.2 ± 1.1 , $n=24$, $N=4$, Figure 5D, top panel). However, in the distal 50 μm of the dendrite (bottom panel), there were no changes in spine numbers across the various treatments (Sham–Control: 84 ± 1.5 , $n=25$, $N=4$; Sham–Stress: 86.3 ± 2.2 , $n=24$, $N=4$; ADX–Veh inj +Stress: 82.5 ± 1.7 , $n=24$, $N=4$; ADX–CORT inj +Stress: 81.8 ± 1.7 , $n=24$, $N=4$). Significant differences in the proximal dendrite are indicated as follows: $*p < .05$, $***p < .001$.

Figure 6.

Increase in anxiety-like behavior is correlated with elevated spine density in basolateral amygdala (BLA) across a range of stress and glucocorticoid manipulations. **(A)** Acute stress increases anxiety (upward arrow) in rats with or without oral vehicle (Veh; white and red squares). Both these groups also have higher spine density on proximal dendrites of BLA neurons (rightward arrow). However, in rats receiving oral CORT overnight (green square), the anxiety index was at control, unstressed levels (black triangle). These two groups with lower anxiety indices also exhibit lower BLA spine density. **(B)** Stress causes higher anxiety index in sham-operated animals (red square). Vehicle (Veh) injection (inj) fails to protect adrenalectomized (ADX) rats against stress-induced increase in anxiety (white square). Both of these groups with high anxiety index (upward arrow) also have higher BLA spine density (rightward arrow). In contrast, in ADX animals, CORT treatment prevents the increase in anxiety and BLA spine numbers (green square) such that both indices are at Sham-Control levels (black triangle). Here too, the lower anxiety indices are accompanied by lower BLA spine density.